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MIXING TWO MONOCLONAL ANTIBODIES YIELDS ENHANCED AFFINITY FOR ANTIGEN[1]

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We observed that mixing monoclonal antibodies directed against various epitopes of human chorionic gonadotropin (hCG) can increase the sensitivity of antigen-binding assays. Depending on the antibody pair chosen, the affinity of the mixture was as much as 10-fold higher than either of the monoclonal antibodies assayed separately. Because hCG does not have any repeating sequences, these results do not reflect a change in avidity of an individual antibody. The increased avidity of the mixture can be detected in both solid-phase assays and liquid-phase double antibody radioimmunoassays. This was not a general property of all monoclonal antibodies raised against hCG. Certain antibodies, apparently reacting with the same region of the antigen molecule, do not bind simultaneously and do not result in enhanced affinity when mixed. In addition, certain other pairs of antibodies do not bind cooperatively even though they can bind simultaneously. The mechanism for the increase in affinity depends on the formation of a multicomponent complex. If one of the antibodies of a pair that results in enhanced affinity upon mixing is replaced by its F(ab) fragment, the enhancement is no longer detectable, indicating it is unlikely the enhancement is due to an allosteric effect. Although the F(ab')$_2$ fragment shows some enhancement when mixed with another antibody, it is not as effective as the intact antibody.

The development of hybridoma antibody technology (1, 2) has provided immunoglobulin reagents that bind to only one antigenic site. Although the utility of these reagents has frequently been limited by their lower affinity for antigen compared with that of serum antibodies (2), in principle, the affinity of monoclonal antibodies could be enhanced by more stringent hybridoma selection procedures. The production of monoclonal antibodies has also enabled investigators to dissect the humoral immune response into its individual components (3). This will eventually result in a more comprehensive understanding of the role of the individual antibody especially with regard to the possibility that an antiserum may have as yet unappreciated characteristics, different from the sum of the individual antibodies.

We have been investigating the immunochemistry of human chorionic gonadotropin (hCG),[2] including the relative orientation of different epitopes of this molecule and the effect of several monoclonal antibodies on the hormone-receptor interaction (4). During the course of the systematic assessment of these antibodies, we observed that the apparent affinity of certain mixtures of monoclonal antibodies is increased relative to the affinity of the individual antibodies. Because hCG has no repeating sequences, this observation could not be related to a change in avidity of individual antibody molecules, but must result from the difference between the affinity of the individual antibodies and the avidity of the mixture. This report contains our data in support of these findings.

MATERIALS AND METHODS

Production of monoclonal antibodies. BALB/c mice were immunized monthly with an i.p. injection of hCG subunits in complete Freund's adjuvant for several months according to the method of Wands and Zurawski (5). Three days before the spleen was excised, additional antigen was injected (50 μg, i.v., in saline).

The spleen cells were fused with P3-NS1/1-Ag4-1 myeloma cells and hybridoma cells were isolated essentially as previously described (5, 6). Antibodies used for subsequent studies were isolated from hybridoma cell lines that were cloned twice by limiting dilution on BALB/c 3T3 monolayers. The antibodies are named with a letter and three numbers. The letter B indicates the antibody is specific for the β subunit of hCG and A indicates the antibody binds the α subunit. All of the antibodies used in this study were IgG1 as determined by radioimmunoassay (reagents purchased from Litton Bionetics, Inc., Kensington, MD).

Cell supernatant, partially purified, and/or purified antibody were used. To obtain partially purified antibody the hybridoma cells were grown in serum-free medium containing 2 mg/ml bovine serum albumin (BSA) and the supernatant was dialyzed against 0.05 M ammonium bicarbonate. After lyophilization, the powder was reconstituted in the desired volume of 0.3 M potassium phosphate buffer, pH 7.5. To obtain purified antibody the same protocol was followed (except the medium contained 0.1 mg/ml BSA), and the albumin was removed with a DEAE Affi-Gel Blue column (Bio-Rad Laboratories, Richmond, CA).

Double antibody radioimmunoassay. Fifty microliters of ^{125}I-hCG and 50 μl of unlabeled hCG (both in 1% horse serum, 99% phosphate-buffered saline) were mixed with 100 μl 0.3 M potassium phosphate (pH 7.5). Subsequently, 100 μl antibody (diluted in 1% horse serum) were added, the tubes were incubated 1 hr at 37°C followed by 18 hr at 5°C, and the complex was precipitated by adding 10 μl of 50% normal mouse serum (in phosphate-buffered saline) and an appropriate amount of rabbit anti-mouse IgG or goat anti-mouse F(ab')$_2$. Once precipitation was complete (i.e., 10 min at 37°C and then 1 hr at room temperature), the precipitate was sedimented and counted.

Solid-phase radioimmunoassay. To coat plastic microtiter wells with antibody, 50 μl purified antibody were incubated for 18 hr at 5°C in a Cooke microtiter plate ("U" wells), and excess nonbound antibody was removed by washing the wells three times with distilled water. To saturate all the plastic sites that would bind proteins, the plates were treated with 10% γ-globulin-free horse serum (90% phosphate-buffered saline) for 2 hr and the excess was removed with distilled water. hCG binding was quantified by adding 50 μl ^{125}I-hCG in 1% horse serum containing varying amounts of hCG. After an incubation of 18 hr at 5°C, excess radioactivity was removed, the plates were washed with distilled water, and the wells were counted.

Sandwich assay. Fifty microliters containing at least 30 μg/ml of monoclonal antibody were added to the 96-well microtiter plates to permit the antibody to adsorb to the surface of the plastic. After 4 hr at 37°C the solution was removed and the plates were immersed in 150 mM NaCl solution containing 1 mg/ml BSA (BSA-saline) to fill remaining nonspecific adsorption sites on the plastic surface. To saturate the antibodies with hCG, the hormone (1 μg in 50 μl BSA-saline) was added for 2 hr to each microliter well. Under these conditions most of the hCG that became insolubilized was bound to antibody adsorbed to the plastic. Excess hCG was removed by washing the plate in BSA-saline solution. After the addition of radiolabeled

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[2] Abbreviations used in this paper: hCG, human chorionic gonadotropin; NaDodSO$_4$, sodium dodecyl sulfate.

antibody (50,000 to 100,000 cpm in BSA-saline solution for 2 hr at room temperature), the nonbound label was removed by washing the plate in BSA-saline, the microtiter plates were cut apart with scissors, and the radioactivity adsorbed to each well was measured.

Other materials and methods. Papain digestion of antibody B102 was performed by methods previously described (7). $NaDodSO_4$ (sodium dodecyl sulfate) polyacrylamide gel electrophoresis (8) of the digestion products indicated that essentially no complete heavy chain remained. The $F(ab')_2$ fragment of antibody B101 was prepared by pepsin digestion (9), except that the amount of pepsin was 2½% by weight of the amount of antibody. Antibody that remained undigested was removed by incubation with protein A-Sepharose (Pharmacia Fine Chemicals) at pH 8.1 and centrifugation of the protein A-Sepharose-antibody complex. Conditions were similar to those employed by Ey *et al.* (10) except that a batch process instead of column chromatography was employed. $NaDodSO_4$ polyacrylamide gel electrophoresis in the absence of reducing agents revealed only one major band at a m.w. of 105,000. The concentration of hCG was determined by the optical density at 280 nm and amino acid analysis. Chloramine-T was used to iodinate hCG according to the procedure described by Greenwood *et al.* (11).

Scatchard analysis of radioimmunoassay data. Affinity of the antibodies or antibody mixtures for hCG was determined by analyzing the data according to Scatchard (12) as described in detail by Moyle (13). The amount of antibody-bound radiolabel (in cpm) divided by the unbound "free" radiolabel (in cpm) was plotted *vs* the concentration of bound hCG (radiolabeled hCG plus the amount of unlabeled hCG). Unbound radiolabeled hCG was computed by subtracting the antibody bound cpm from the total cpm that could be bound by antibody. Total bindable cpm was determined by adding an excess of antibody or plotting 1/antibody dilution *vs* 1/cpm bound and extrapolating to infinite antibody concentration.

RESULTS

Radioimmunoassays. We compared the ability of hCG to inhibit the binding of radioiodinated hCG to monoclonal antibodies B101, B102, and a mixture of B101 and B102 as shown in th radioimmunoassays of Figure 1*A*. HCG was 10-fold more active in inhibiting binding of radiolabel when the 1:1 mixture was employed. Scatchard analysis of the data confirmed this was du to an increase in affinity (Fig. 1*B*, *C*, and *D*). The gr ater affinity of the mixture enabled us to dilute a 1:1 mixture of th two antibodies ninefold (each individual antibody was first diluted so that about one-half the radiolabeled hCG could be bound) and achieve the same amount of tracer binding as occurred with the same dilution of the separate antibodies. These r sults were highly reproducible, as shown in Table I. Although the data are not presented here, the ability of two antibodies to show this cooperative effect is not limited to pairs of antibodies that bind the same subunit—A102 and B102 also xhibit this cooperative interaction. Other combinations of antibodi s to hCG did not produce the same result. Thus, we did not observe a similar cooperative effect between B101 and A102 (Fig. 2*A*) or between B101 and B103 (Fig. 2*B*). Mixtures of B101 and A102 (or B101 and B103), for example, gave inhibition curves intermediate between those antibodies. Similar results are apparent with a solid-phase radioimmunoassay (Fig. 3). We observed that a mixture of B101 and B102 had a high r affinity for hCG than either of those antibodies exhibited separately, although the difference between the mixture and antibody B101 was only about fourfold. The combination of antibody B102 and B103 did not result in an increase in affinity. H nc , this phenomenon was present in some but not all mixtur s of antibodies.

Sandwich assays. To obtain data bearing on the mechanism of th affinity change, we measured the ability of the antibodies to bind to different sites on hCG by sandwich assays (Table II). If unlab led and radiolabeled antibody bind to the same site, th unlabeled antibody will inhibit binding of the radiolabel. Conv rsely, if both bind to differ nt sit s, a large amount of radiolabel will be bound to the plastic. Labeled and unlabel d B101 cannot bind the antigen at th sam time; th ref re, w observed that minimal radiolabel bound to the plastic. We observed similar results using labeled and unlabeled B102. We observed that antibodies B102 and B103 bind to hCG at a sit remote from that for B101 because both allow binding of B101 to the plastic. Further, labeled B102 bound to hCG attach d to unlabeled B101. In contrast, antib dy A102 pr vent d binding of radioactive B101, indicating that A102 and B101 cannot bind simultaneously to hCG. Similarly, w found that antibodies

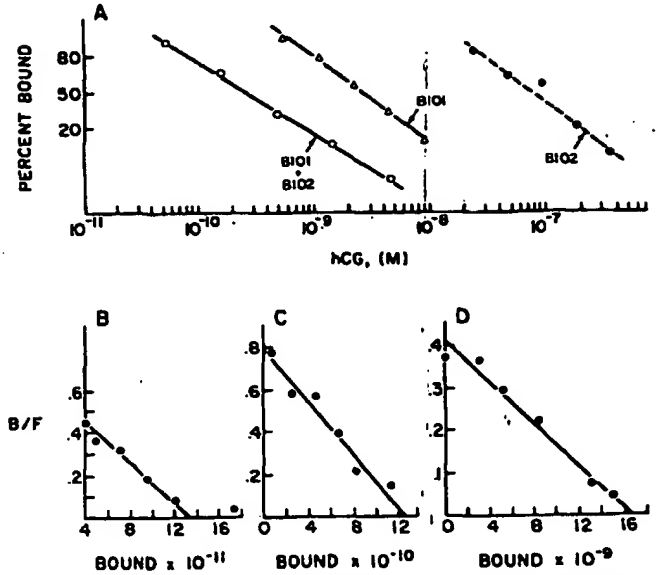

Figure 1. A, radioimmunoassay for hCG by double antibody liquid-phase assay. The amount of labeled hCG bound in the absence of unlabeled inhibitor was approximately the same for each antibody or antibody mixture. This was achieved by appropriate dilution of each antibody solution or mixture. Rabbit anti-mouse IgG was the second antibody. The ED_{50} (effective displacement of 50% of bound radiolabeled hCG) ± SD was calculated for each antibody or mixture. The ED_{50} for the mixture of B101 and B102 is $2.44 \pm 0.74 \times 10^{-10}$ M, antibody B101 $2.88 \pm 0.74 \times 10^{-8}$ M, and antibody B102 $1.058 \pm 0.995 \times 10^{-7}$ M. *B, C, D,* Scatchard analysis of the binding to hCG of B-mixture of antibodies B101 and B102, C-antibody B101, and D-antibody B102. Slopes of the lines (therefore, the equilibrium binding constants) are *B*, 5.4×10^{9}, *C*, 5.1×10^{9}, and *D*, 1.9×10^{7}. The mols of each antibody can be inferred from the intercept on the abscissa of the Scatchard plots. Thus, the concentrations of B101 and B102 (from Figures 1*C* and 1*D*, assuming bivalent antibodies) are, respectively, 6.5×10^{-10} M and 8×10^{-9} M. The data in Figure 1*B* indicate that the concentration of the high affinity sites is 1.3×10^{-10} M. The concentration of B101 and B102 added to obtain the results in Figure 1*B* were 3.6×10^{-11} M and 4.4×10^{-10} M, respectively.

TABLE I

Statistical analysis of the affinity of antibodies B101 and B102 compared with a mixture of antibodies B101 plus B102

Expt. No.	Antibody or Mixture	Keq[a]	Enhancement Factor[b]	Probability
		liters/nmol		
1	B101	0.51 ± 0.02		
	B102	0.019 ± 0.002		
	B101 + B102	5.4 ± 0.5	10.6	<0.01
2	B101	0.41 ± 0.09		
	B102	0.018 ± 0.002		
	B101 + B102	3.0 ± 0.2	7.3	<0.01
3	B101	0.88 ± 0.08		
	B101 + B102	5.0 ± 0.07	5.7	<0.01
4	B101	0.81 ± 0.11		
	B101 + B102	5.5 ± 1.2	6.0	<0.01

[a] Determined by using Scatchard plots (12).
[b] Keq for a mixture of B101 and B102 divided by Keq for B101. For the purposes of calculating the enhancement, the affinity of the mixture is compared to the affinity of the antibody with the higher affinity.